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Fatty acid compositions of red blood cell phospholipids in children with autism ☆

B. Bu[a,1], P. Ashwood[b,1], D. Harvey[c], I.B. King[d], J.Van de Water[e], L.-W. Jin[a,*]

[a]*Department of Pathology, M.I.N.D. Institute, University of California at Davis, 2805 50th Street, Sacramento, CA 95817, USA*
[b]*Department of Medical Microbiology and Immunology, M.I.N.D. Institute, NIEHS Center for Children's Environmental Health, USA*
[c]*Department of Public Health Sciences, Division of Biostatistics, University of California at Davis, CA, USA*
[d]*Public Health Sciences Laboratories, Fred Hutchinson Cancer Research Center, Seattle, WA, USA*
[e]*Department of Internal Medicine, Division of Rheumatology, Allergy and Clinical Immunology, MIND Institute, NIEHS Center for Children's Environmental Health, University of California at Davis, CA, USA*

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Abstract

We compared the compositions of fatty acids including n-3, n-6 polyunsaturated fatty acids, *trans*- and *cis*-monounsaturated fatty acids, and saturated fatty acids in the red blood cell membranes of 40 children with autism (20 with early onset autism and 20 with developmental regression) and age-matched, 20 typically developing controls and 20 subjects with non-autistic developmental disabilities. The main findings include increased levels of eicosenoic acid (20:1n9) and erucic acid (22:1n9) in autistic subjects with developmental regression when compared with typically developing controls. In addition, an increase in 20:2n6 and a decrease in 16:1n7t were observed in children with clinical regression compared to those with early onset autism. Our results do not provide strong evidence for the hypothesis that abnormal fatty acid metabolism plays a role in the pathogenesis of autism spectrum disorder, although they suggest some metabolic or dietary abnormalities in the regressive form of autism.

1. Introduction

Autism is a neurodevelopmental disorder usually diagnosed before 3 years of age, and is clinically characterized by impairments in socialization, verbal and non-verbal communication, and stereotyped, restricted and repetitive behaviors [1]. Little is known about the etiology of autism. Abnormalities in the fatty acid compositions of phospholipids, the major constituents of cell membranes, have been implicated in several neurodevelopmental disorders that manifest with psychiatric symptoms. For example, in schizophrenia, changes of red blood cell (RBC) membrane phospholipids such as deficiencies in n-3 polyunsaturated fatty acids (PUFA) have been reported [2–5]. It has been proposed that the defects in PUFA could play a role in the pathophysiology of schizophrenia. Indeed, supplementing diets with fish oil was shown to correct these deficiencies and lead to improvements in the symptom scores of schizophrenic patients [6].

Similarly, defects of fatty acids and phospholipids have recently been reported in autism subjects, including not only reduced levels of n-3 PUFA, but also increased levels of saturated fatty acids (SFA) in the RBC membrane [7,8] or in plasma [9]. Further evidence from Bell et al. suggested that decreased levels of arachidonic acid (ARA), docosatetraenoic acid (DTA) and docosahexaenoic acids (DHA) in RBC membranes from autism subjects could be caused by increased activity of RBC

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*Corresponding author. Tel.: +1 916 703 0392; fax: +1 916 703 0370.
E-mail address: lee-way.jin@ucdmc.ucdavis.edu (L.-W. Jin).
[1]These authors equally contributed to this work.

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type IV phospholipase A2, suggesting that altered metabolism of phospholipids may occur in autism [8]. In addition, some of the problems that have been reported in autism, such as excessive oxidation or decreased dietary supplies of essential fatty acids may contribute to fatty acid deficiencies [10–12].

In view of the vital role of fatty acids in neural membrane function and cell homeostasis, the defects in fatty acid metabolism may have important biological and etiological implications in autism. In addition, the fatty acid profiles of RBC, a sample that can be obtained in routine clinical practice, could potentially be used for diagnostic purposes, if validated. To investigate whether abnormalities in membrane fatty acids are associated with autism, we compared the compositions of fatty acids in RBC membranes between autistic children, children with developmental disabilities (DD) and typically developing controls.

2. Patients and methods

2.1. Participants

This case-control population-based study examined 80 children, enrolled through the Center for Children's Environmental Health and the Medical Investigations of Neurodevelopmental Disorders (M.I.N.D.) Institute as part of the ongoing Childhood Autism Risk from Genetics and Environment (CHARGE) study at UC Davis. The CHARGE study population is sampled from three strata: children with autism, age-matched children selected from the general population with typical development, and children with mental retardation or DD but without autism. Inclusion criteria include children born in California, between 24 and 60 months of age at the time of assessment, living with at least one biologic parent who is English or Spanish speaking, and residence within areas covered by participating Regional Centers in northern and southern California, which determines eligibility for California State-funded developmental services. It is estimated that at least 75–80% of the total population of children with an autism diagnosis in the state are enrolled in these Regional Centers [13].

To confirm and further detail the initial diagnosis, all children were assessed at the UC Davis M.I.N.D. Institute. Autism was confirmed for all cases using the Autism Diagnostic Interview-Revised (ADI-R) [1] and the Autism Diagnostic Observation Schedule, modules 1 and 2 (ADOS) [14–17]. The ADI-R provides a standardized, semi-structured interview and a diagnostic algorithm for the DSM-IV [18] and the ICD-10 definitions of autism [19,20]. The ADOS is a semi-structured, standardized assessment in which the researcher observes the social interaction, communication, play and imaginative use of materials for children suspected of having autism. Final autism case diagnosis was defined as meeting criteria on the communication, social, and repetitive behaviors domains of the ADI-R and scoring at or above the cut off for autistic disorder on the ADOS module 1 or 2. The control group children with no DD were identified from State birth certificate files and frequency-matched for age, gender, and obtained from the same geographical residential areas as for the regional center distribution of the autism cases. Children with developmental delay or mental retardation were recruited through the same regional centers as the autism cases. The Social Communication Questionnaire [21] was used to screen for behavioral and developmental characteristics of autism; comparison subjects who scored above the screening cut-off were eliminated from the study.

All children were assessed with the Mullen Scales of Early Learning [22] and the Vineland Adaptive Behavior Scales [20] to assess developmental and adaptive function, and examined by a developmental–behavioral pediatrician for dysmorphic, physical or neurologic variations. Particular emphasis was placed on documentation, through history and record review of developmental regression or loss of previously acquired developmental skills.

The sample population included children diagnosed with autism ($n = 40$, median age 3.6 years, range 2.1–5, three females), age-matched typically developing controls ($n = 20$, median age 3.5 years, range 2.3–5, four females), and children diagnosed with DD ($n = 20$, median age 3.1 years, range 2–4.6, three females). The children with autism were further subdivided based on their ADI scores into early onset autism ($n = 20$, median age 3.4 years, range 2.1–4.8, one females), and delayed onset (regressive) autism ($n = 20$, median age 3.5 years, range 2.5–5, two females). The children in the early onset group had delays in the development of early skills, while those in the delayed onset group had developed some language or social skills that were subsequently lost, usually between 18 and 24 months of age. The study protocol followed the ethical guidelines of the most recent Declaration of Helsinki [23], and was approved by the Institutional Review Board. All subjects enrolled in the study had written informed consent provided by their parents and assented to participate if developmentally able.

Upon survey, 22 children with autism (55%) and 9 typically developing controls (45%) in the group were taking supplementary Gummy Bear multivitamins or Flintstone multivitamins (Table 1).

2.2. Blood sampling

For both control and autism groups, blood samples were collected in the afternoon (typically between 2 and

Table 1
Participants' dietary supplements

Dietary supplements	Autism ($n = 40$)	Typically developing ($n = 20$)
Multiple vitamins*	22 (55%)	9 (47.75%)
Melatonin	4	0
Cod liver oil	1	0
Enzyme supplement	1	0
Calcium	2	0

*No significant difference between autism and typically developing controls ($P = 0.6$, χ^2-test).

4 pm) from subjects after they had finished behavioral assessments. The parents were instructed not to give any food to the children for at least 2 h before the blood draw. Eight milliliters of venous blood were drawn in ACD solution Vacutainers (BD Biosciences, San Jose, CA, USA), and centrifuged for 10 min at 2300 rpm at room temperature and the plasma was collected. The cellular part was then mixed 1:1 with Hanks balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD, USA) without Ca^{2+} or Mg^{2+}. The diluted cells was layered carefully over a Ficoll-Paque gradient (Pharmacia Biotech, Piscataway, NJ, USA) and centrifuged at 2300 rpm for 30 min at room temperature. Mononuclear cells were harvested from the interface layer. The remaining RBC pellet was collected, washed twice with HBSS, and stored at −80 °C until analysis.

2.3. Fatty acid extraction and esterification

Fatty acids were extracted from RBC by method of Rose and Oklander [24]. The cells were promptly thawed and an aliquot of 0.25 ml was mixed with an equivalent volume of distilled/deionized water and processed as described previously [25]. The fatty acid methyl esters (FAME) samples were prepared by direct transesterification using the method of LePage and Roy [26].

2.4. Gas chromatography

Gas chromatography was performed on samples as described by Lamaitre et al. [27] with few minor modifications. Briefly, samples were dissolved in hexane. The FAME of individual fatty acids in RBC were separated on a gas chromatograph (model 5890B, series II, Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID), automatic sampler (model 7673, Hewlett-Packard), electronic pressure programming (EPP) (Hewlett-Packard), and Chemstation software (Hewlett-Packard). FAMEs were separated on a SP-2560 wall-coated open-tubular fused silica capillary column, 100 m × 0.25 mm ID, 0.20 μm film thickness (Supelco, Bellefonte, PA, USA). The carrier gas, helium, was set at 60 pounds per square inch (PSI) at the tank; the make-up gas, nitrogen, was also set at 60 PSI. At the detector, hydrogen was set at 30 PSI and breathing air at 20 PSI. The initial column velocity was set at 20 cm/s (oven temperature 200 °C). The injector port temperature was set at 250 °C and detector port temperature was set at 275 °C. The oven temperature (165 °C at the start) and electronic pressure (50 PSI at the start) was controlled by a set program for a total run of 117 min to optimize *trans*-fatty acids separations. Quantitative precision and identification were evaluated using model mixtures of known FAMEs and an established control pool. Fatty acids are expressed as relative weight percents. Interassay coefficients of variations (CVs) were on the average 2.5% or lower for most of the fatty acids that are present at levels of 1% or higher. Specifically, interassay CVs were 2.2%, 8.0%, 2.0% and 1.3% for 20:1n-9, 22:1n-9, 16:1n-7t, and 20:2n-6, respectively.

2.5. Statistical analysis

Prior to analyses, we used graphical approaches including histograms and normal quantile–quantile plots to illustrate the distributions of the fatty acid levels, to detect outliers, and to assess the variability in the fatty acid levels within the different groups of subjects. Due to the skewness of the distributions, many of the fatty acid levels were transformed using the natural logarithm transformation for use in the analyses. Two-sample *t*-tests were used to compare the means between pairs of groups. In addition, the non-parametric Wilcoxon rank sum test was used to assess differences in the distribution of the fatty acid levels between pairs of groups, particularly for the fatty acids that still violated the normality assumption of the *t*-test even after transformation. Due to the exploratory nature and hypothesis generating purpose of this analysis and study, no correction for multiple comparisons was made. Therefore a *P*-value of less than 0.05 was considered statistically significant. All *P*-values reported are from the *t*-test, unless otherwise stated.

3. Results

The results from our lipid analysis are presented in Table 2. Our data do not confirm previously reported deficiencies of n-3 and n-6 essential fatty acids in autism (*P*-values ranging from 0.06 to 0.98, *t*-test or Wilcoxon rank sum tests). The ARA/eicosapentaenoic acid (EPA) ratio, reported by Bell et al. to be significantly increased in both regressive autism and Asperger's syndrome patients [8], does not display any significant change between groups in our study. In addition, our data did not confirm the changes in the levels of total saturated

Table 2
Compositions of fatty acids in RBC membrane in subject groups

Fatty acids	Autism ($n = 40$)	Early onset autism ($n = 20$)	Regressive autism ($n = 20$)	Developmental disabilities ($n = 20$)	Typically developing ($n = 20$)
SFA					
14:0	0.24±0.10	0.26±0.10	0.21±0.09	0.26±0.09	0.23±0.05
15:0	0.12±0.05	0.14±0.05	0.11±0.04	0.13±0.05	0.12±0.02
16:0	19.7±1.9	19.8±2.1	19.6±1.7	20.0±2.9	19.9±1.7
17:0	0.34±0.06	0.35±0.05	0.33±0.07	0.36±0.07	0.35±0.04
18:0	14.17±0.76	14.25±0.72	14.08±0.80	14.65±1.35	14.32±0.94
20:0	0.40±0.07	0.41±0.07	0.39±0.07	0.42±0.08	0.41±0.06
22:0	2.1±04	2.1±0.4	2.0±0.5	1.9±0.5	2.1±0.3
24:0	4.55±0.90	4.45±0.91	4.65±0.90	4.38 ±1.02	4.59±0.54
Total SFA	41.57±3.57	41.76±3.72	41.38±3.50	42.03±5.47	42.02±3.21
MFA, cis bonds					
14:1n5	0.006±0.005	0.006±0.005	0.006±0.005	0.004±0.005	0.004±0.005
16:1n7	0.24±0.10	0.26±0.11	0.22±0.07	0.26±0.13	0.24±0.07
16:1n9	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01	0.04±0.01
17:1n9	1.04±0.24	1.00±0.21	1.08±0.27	1.04±0.23	0.96±0.15
18:1n5	0.73±0.37	0.73±0.32	0.73±0.43	0.58±0.25	0.69±0.18
18:1n7	0.89±0.15	0.87±0.15	0.90±0.14	0.85±0.12	0.88±0.11
18:1n8	0.11±0.04	0.11±0.04	0.11±0.04	0.10±0.03	0.11±0.02
18:1n9	11.64±0.95	11.47±0.96	11.81±0.93	11.61±1.29	11.28±0.93
20:1n9	0.25±0.07*	0.23±0.07	0.27±0.07*	0.23±0.06	0.22±0.03
22:1n9	0.07±0.02	0.07±0.02	0.07±0.02*	0.06±0.01^	0.06±0.01
24:1n9	4.25±0.97	4.14±1.07	4.36±0.89	4.18±0.90	3.98±0.62
Total MFA, cis bonds	19.26±1.89	18.94±2.09	19.59±1.67*	18.94±2.02	18.47±1.31
PUFA, n-3					
18:3n3	0.11±0.03	0.11±0.03	0.11±0.02	0.12±0.07	0.11±0.03
20:3n3	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.005
20:5n3 (EPA)	0.30±0.47	0.35±0.55	0.26±0.39	0.22±0.12	0.21±0.14
22:5n3	1.54±0.60	1.63±0.65	1.45±0.54	1.53±0.52	1.46±0.40
22:6n3 (DHA)	2.12±1.38	2.22±1.54	2.02±1.24	1.81±0.64	2.17±1.00
Total n-3 PUFA	4.10±2.28	4.33±2.56	3.86±2.00	3.71±1.14	3.98±1.48
PUFA, n-6					
18:2n6	9.92±1.29	9.89±1.32	9.96 ±1.29	9.63±2.01	10.16±1.59
18:3n6	0.04±0.01	0.04±0.01	0.04±0.02	0.05±0.02	0.04±0.02
20:2n6	0.25±0.05	0.24±0.05@	0.27±0.05@	0.26±0.09	0.25±0.05
20:3n6	1.51±0.44	1.53±0.44	1.49±0.45	1.50±0.50	1.42±0.21
20:4n6 (ARA)	12.39±2.47	12.35±2.58	12.43±2.43	12.94±3.12	12.55±2.28
22:2n6	0.08±0.02	0.07±0.03	0.08±0.02	0.08±0.03	0.08±0.02
22:4n6	3.5±0.8	3.4±0.8	3.6±0.8	3.7±0.9	3.6±0.8
Total n-6 PUFA	27.69±3.82	27.55±4.09	27.84±3.63	28.13±5.34	28.01±3.15
Unsaturated FA, trans bonds					
16:1n7t	0.13±0.05	0.15±0.04@	0.12±0.05@	0.13±0.05	0.14±0.03
16:1n9t	0.05±0.02	0.05±0.01	0.06±0.02	0.04±0.01#	0.05±0.01
18:1n10-12t	0.18±0.07	0.18±0.07	0.19±0.08	0.15±0.05	0.16±0.04
18:1n9t	0.30±0.13	0.29±0.12	0.31±0.15	0.24±0.09	0.28±0.06
18:1n8t	0.64±0.29	0.63±0.27	0.65±0.31	0.52±0.19	0.61±0.13
18:1n7t	0.50±0.20	0.50±0.18	0.50±0.22	0.41±0.13	0.47±0.09
18:1n6t	0.60±0.19	0.58±0.18	0.61±0.21	0.51±0.14	0.56±0.10
18:2n6tt	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
18:2n6ct	0.07±0.02	0.07±0.02	0.07±0.03	0.06±0.01	0.07±0.01
18:2n6tc	0.09±0.03	0.09±0.03	0.09±0.04	0.08±0.02	0.08±0.02
Total *trans*-unsaturated FA	2.58±0.95	2.56±0.86	2.61±1.05	2.16±0.63	2.44±0.44
ARA/EPA ratio	71.69±35.95	66.43±40.75	76.94±30.56	66.40±21.31	72.43±23.09

The values are wt% of total fatty acids and expressed as average±standard deviation.
* $P<0.05$ when compared with typically developing controls.
@ $P<0.05$ when compared between classic and regressive groups.
# $P<0.02$ when compared with both autism subjects and typically developing controls.
^ $P<0.05$ when compared with regressive autism group.

and monounsaturated fatty acids (MFA) in autism patients reported by Bell et al. [8] and Vancassel et al. [9].

However, we observed a few previously unreported alterations in fatty acid levels. The percentage of eicosenoic acid (20:1n9) was significantly increased in children with autism compared with typically developing controls ($P = 0.02$). Among children with autism, those with regressive autism, but not those with early onset autism, showed a significantly higher percentage of eicosenoic acid compared with typically developing controls ($P = 0.01$), indicating that the increase seen in the autism subjects mostly came from the increase in the regressive autism group. However, the eicosenoic acid percentage in children with regressive autism was not significantly different from that in children with early onset autism ($P = 0.13$), or in DD controls ($P = 0.08$). Interestingly, the level of erucic acid (22:1n9, an elongated form of eicosenoic acid) in the regressive autism group was also significantly increased when compared with typically developing controls ($P = 0.032$, Wilcoxon rank sum test) or with the DD group ($P = 0.04$, Wilcoxon rank sum test), but not different from that in the children with early onset autism. The total level of *cis*-MFA was also significantly increased in the regressive group when compared to the typically developing controls ($P = 0.023$). Furthermore, comparisons between the two autism groups showed that the percentage of 11,14-eidosadienoic acid (20:2n6) was significantly elevated ($P = 0.03$), and the percentage of a *trans*-unsaturated fatty acid 16:1n7t was significantly decreased in regressive autism subjects compared with early onset autism subjects ($P = 0.04$, Wilcoxon rank sum test). However, there was no difference in these two fatty acids between autism children and DD or typically developing controls ($P > 0.2$).

In the DD group, palmitelaidic acid (16:1n9t) was significantly reduced when compared to both autism subjects and typically developing controls ($P = 0.02$, Wilcoxon rank sum test, for both comparisons). This reduction appears to be unique to the DD group, since no significant changes were observed in either regressive or early onset autism patients when compared to typically developing controls.

4. Discussion and conclusion

We report a few alterations in the RBC fatty acid compositions of subjects from the CHARGE study, a case-control, population-based study. We found increased levels in both eicosenoic and erucic acids, as well as in total cis bond MFA in children with regressive autism when compared to the typically developing controls. Also, when compared to children with early onset autism, the children with regressive autism had an increased level of 20:2n6 and a decreased level of 16:1n7t. For exploratory purposes in our analysis, we did not correct for multiple comparisons when testing for differences between groups. None of the results would be significant if a correction had been applied.

A similar study by Bell et al. [8] reported that patients with regressive autism had higher percentages of 18:0, 18:2n-6 and total saturates in their RBC membranes compared to controls, while 24:0, 22:5n-6, 24:1 and the ARA/EPA ratio were higher in both regressive autism and early onset autism/Asperger's syndrome cases compared to controls. Conversely, the 18:1n-9 and ARA values were lower in patients with regressive autism compared to controls while 22:5n-3, total n-3 and total dimethyl acetals were lower in both regressive autism and early onset autism/Asperger's groups compared to controls. Since similar abnormalities have been described in a number of other neuropsychiatric disorders such as schizophrenia, attention-deficit/hyperactivity disorder, depression and bipolar disorder [2,28–31], the reported findings imply that a mechanism involving abnormal membrane phospholipids may be shared by these neuropsychiatric disorders.

All these abnormalities reported by Bell et al. [8], however, were not replicated in the present study. The reason for this discrepancy is not clear. Comparing to Bell et al. our sample size is slightly larger. Our subjects, 24–60 months of age, were age-matched between groups, while the ages of subjects in Bell et al. were not described and not age-matched between groups. In addition, different from Bell et al. our samples were strictly stored at $-80\ ^{\circ}C$ to avoid oxidation. One of the aims of the CHARGE study is to investigate whether the occurrence of autism is related to food habits. For example, intake of fish rich in n-3 fatty acids may significantly affect the lipid compositions of phospholipids [32]. A survey of the food questionnaires that were obtained from all cases did not reveal any significant differences of dietary supplements including fish oils between groups. However, the EPA (20:5n3) levels in both classic and regressive autistic groups had wider variations compared to the DD and the typically developing group (Table 2). The reason for this observation is not clear, but it might be the result of some food preference of children with autism.

Our results show differences among groups in the levels of a few low-abundance fatty acids. The levels of two PUFA, eicosenoic acid and erucic acid, were elevated in children with regressive autism compared to typically developing controls. Both are elongation products of 18:1n9. The levels of other elements of this synthetic pathway such as 18:1n9 and 24:1n9 were not changed in autistic subjects. Eicosenoic acid is an ingredient rich in canola oil. Canola oil is not approved for use in infant formula due to the concerns over possible accumulation of triglyceride in the heart as a

result of the small amount of erucic acid in this oil [33–35]. Regular canola oil used in households is derived from plants genetically engineered to have low levels of erucic acid [36]. The increases of these two fatty acids, therefore, might be a result from a unique dietary factor not revealed by the food survey. Alternatively, they could be due to in vivo fatty acid elongation as a result of some metabolic disturbance. Interestingly, the increases in the levels of eicosenoic and erucic acid were not observed in the early onset autism subjects, suggesting that the event leading to their increases might be related to the developmental regression.

The 20:2n6 is the elongation product of 18:2n6. It has been suggested that a higher level of 20:2n6 can result from diets high in n-6, especially 18:2n6 [37]. The biological significance of the differences in the percentages of 20:2n6 and 16:1n7t observed in regressive autism compared with early onset autism is not clear, especially since no differences were found when compared to typically developing controls.

Although confirmation by future studies using larger sample sizes and other sample types are necessary, our results, in contrast to previous reports [7,8], do not provide strong evidence for the hypothesis that autism is a disorder of fatty acid metabolism [38]. Our conclusion is consistent with a previous report demonstrating no evidence of altered phosopholipid-related signal transduction (as expected in membrane phospholipids disorders) in autism as assessed by the niacin skin flush test [39]. Since niacin skin response is reduced in schizophrenia, it appears that fatty acid abnormalities in autism, if any, are likely to differ from those in schizophrenia. We report a few alterations of RBC membrane fatty acids in regressive autistic children. Although these changes must be considered with caution due to the relatively small sample size, they do suggest that regressive form of autism is associated with some metabolic or dietary fatty acid abnormalities. Whether a combination of these fatty acid alterations could be used for the chemical diagnosis of autism with developmental regression awaits further studies.

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